CASE REPORT

A *De Novo* Novel Mutation of the *EDNRB* Gene in a Taiwanese Boy with Hirschsprung Disease

*Wen-Chau Chen, Shen-Shun Chang,[1] Edgar D. Sy,[1] Ming-Che Tsai**

Hirschsprung disease (HSCR) is a congenital disorder characterized by an absence of ganglion cells in the nerve plexuses of the lower digestive tract. Although mutations in eight different genes (*EDNRB*, *EDN3*, *ECE1*, *SOX10*, *RET*, *GDNF*, *NTN*, *SIP1*) have been identified in affected individuals, it is now clear that *RET* and *EDNRB* are the primary genes implicated in the etiology of HSCR. All eight genes are involved in the early development of the enteric nervous system, and most act through two distinct biochemical pathways mediated by *RET* and *EDNRB*. Mutations in *RET* and *EDNRB* account for up to 50% and 5% of HSCR cases in the general population, respectively. Interaction between these two signaling pathways could modify *RET* expression and, therefore, HSCR phenotype. Here, we report the case of a 1-year-old Taiwanese boy who presented with abdominal distension since birth and bilious vomiting after feeding. HSCR (short-segment type) was diagnosed based on X-ray, lower gastrointestinal series and biopsy findings. Mutation analysis revealed a heterozygous T>C missense mutation in exon 1 of the *EDNRB* gene, that substitutes the highly conserved cysteine-90 residue in the extracellular domain of the G protein-coupled receptor with an arginine residue (C90R). No *RET* gene mutation was detected in this patient. [*J Formos Med Assoc* 2006;105(4): 349–354]

Key Words: *EDNRB* gene, Hirschsprung disease, Taiwanese

Hirschsprung disease (HSCR, OMIM 142623), or aganglionic megacolon, is a congenital disorder characterized by the absence of enteric ganglia along a variable length of the intestine. The estimated incidence is approximately 1 in 5000 live births. Molecular genetic analysis has identified several genes that have a role in the development of HSCR; the major susceptibility gene for this disorder is the *RET* proto-oncogene.[1] Genes encoding functional ligands of the RET-receptor complex, such as the glial cell line-derived neurotropic factor (*GDNF*), neurturin (*NTN*),[2] artemin (*ARTN*), persephin (*PSPN*), and corresponding members of the *GDNF*-family receptor α genes (*GFRα-1-4*),[2] have also been suggested as putative susceptibility genes associated with HSCR. Waardenburg-Shah syndrome is a disorder of the embryonic neural crest that combines the clinical features of Waardenburg syndrome and HSCR.[3] Patients with Waardenburg-Shah syndrome with megacolon have a homozygous founder mutation in the G-protein-coupled endothelin-B receptor gene (*EDNRB*),[4] whereas heterozygous mutations of *EDNRB* and endothelin-3 (*EDN3*)[5] have been identified in individuals with isolated HSCR. Heterozygous mutations of *SOX10* have been described in patients with megacolon in Waardenburg-Shah syndrome.[6]

Mutation of the *RET* gene accounts for up to 20% of sporadic and 50% of familial cases.[7] Mutation of the *EDNRB* gene accounts for 5–10% of all HSCR cases.[8,9] Short-segment HSCR occurs

Departments of Emergency Medicine and [1]Surgery, National Cheng Kung University Medical College and Hospital, Tainan, Taiwan.

Received: March 31, 2005
Revised: May 3, 2005
Accepted: July 5, 2005

***Correspondence to:** Dr. Ming-Che Tsai, Department of Emergency Medicine, National Cheng Kung University Hospital, 138, Sheng-Li Road, Tainan 704, Taiwan.
E-mail: terence39@yahoo.com

in about 25% of *RET*-caused cases and in more than 95% of *EDNRB*-related cases.[8] Even in families with apparent monogenic inheritance, there is incomplete penetrance of disease-causing mutations and intra- and interfamily variation of phenotype severity, suggesting that modifying genetic, stochastic or environmental factors are involved.

In this report, we describe the genetic analysis of the *RET* and *EDNRB* genes in a Taiwanese boy with HSCR.

Case Report

The proband was a 1-year-old Taiwanese boy with HSCR. He presented with abdominal distension since birth and bilious vomiting after feeding. The baby was born at full term with a birth weight of 2665 g. He was the first child of this family. No other family members had a history of the same symptoms/signs.

Physical examination did not show any pigmentary anomalies or deafness. X-ray examination showed diffuse enlarged bowel gas with absent bowel gas in the rectal area. Lower gastrointestinal series showed an enlarged cecum, ascending colon and ileum without focal obstruction sign. Suction biopsy was performed and pathology revealed no ganglial neurons in the rectum and sigmoid colon. Acetylcholine esterase stain was positive. Under the impression of HSCR (short-segment type), colostomy was arranged.

PCR and automated DNA sequencing

Genomic DNA was extracted from the peripheral blood (QIAamp Midi Kit; Qiagen, Valencia, CA, USA) of the proband and parents after obtaining informed consent. DNA samples were then subjected to mutation screening by amplification of segments of the *RET* and *EDNRB* genes with primers (Tables 1 and 2) synthesized on the basis of intronic sequences from Genbank.

Table 1. Polymerase chain reaction primers used for the amplification of the *RET* gene from genomic DNA

Exon	Forward (5' → 3')	Backward (5' → 3')	Product size (bp)	AT (°C)
1	CGGCGCTTACCTCGCTTCAG	TGTCCCGTTTGCTCCAGGAC	622	62
2	CAGTTCTTTTCTAGCCCGTG	ATGATTCCCGTGTGTCTCCA	671	52
3	GTTTACACCAGCCCTGGAGC	GCTCTGTCTGCCCCACAAGA	631	55
4	CTGTGGAGCGGAGGAGGGGA	CTAGGACAGACGGCGCAGAC	534	62
5	CTGACAACACACATCTGGTC	CAGAGACACAGGAAGTGCTG	481	55
6	CGTGTTTGCACCAGTGTGAG	CACCCAGTCTACTCTGTGCT	401	53
7	GTTCCAGGACTTAGGCTGTG	AGCCTTGCAGCTGTACTGCT	449	53
8	CTGGCACTGTCTTTGCTGCC	CTCACAAGCCCTCTCCCAAG	468	55
9	CTCCTCTCCCATAAGCCATG	GAACTGACAGCCCTGGCAAC	391	55
10	CAGAAAGGCACTGTGACCAA	CAGGCTGACAAGTTGTTTGG	554	52
11	GTAAATGGCAGTACCCATGC	CACAGCGCCCTATGGAAATG	592	52
12	GCAGAGACAGGCAGCGTTGC	CTCGCTCTGCTTCTCTAGGC	458	55
13	CTCTCTGTCTGAACTTGGGC	CAGTAGGGAAAGGGAGAAAG	312	52
14	CAGAGCTGCAGCAGTGCTGC	CATGCCATGGCAGGGGCATG	508	52
15	CTGCCATGTCACACCCTGAC	GTCAGTATGCTGCCAGGGAG	540	57
16	CAGGAGTGTCTACAGCACTC	CATTGCAGAGGGCTAGCACT	340	53
17	CGACAGGGTCAGCAGGTGCT	CTGGTTTCTCCTGGGGCTGC	341	58
18	CTTTGGAGTTGGAGACAGAG	CATGACTCTCTCTCTCTGCA	361	50
19	CTGGTCTCTTGGAGAGGTCA	GGTTCAGAGCAGACTTTGGT	411	50
20	CACAGAAACCACGAGTTTGG	CTGCTAGGAGGGAAAATCAC	470	50

AT = annealing temperature.

Table 2. PCR primers used for the amplification of the *EDNRB* gene from genomic DNA

Exon	Forward (5' → 3')	Backward (5' → 3')	Product size (bp)	AT (°C)
1	CTCTGCTTGTCTCTAGGCTC	GATTCAGTAGGTCTGGGGTG	881	55
2,3	GTGATACAATTCAGAGGGCA	CACTGAGATCAAGGGGATTC	734	50
4	CAGTAAGTGTGGCCTGAAAG	GTGAAGTGGAACCGAAGTGA	562	50
5	GATCTAGGGAGAATCAGAAC	GAAGTACTGAAGCTGGCTGA	643	50
6	GCACAGAAGCTACAATGACT	CTACCAAAAACAGGGAACAG	530	50
7	CAAAGAAAGTCAGAACCCTG	TCCATGCCGTAAACAGCTCA	407	50

AT = annealing temperature.

For polymerase chain reaction (PCR) amplification, approximately 200 ng of genomic DNA, 12.8 pmol of each primer, 10 μmol dNTP and 1.25 U of Taq (Qiagen) were used in a total volume of 50 μL. The amplification conditions were 94° C for 5 minutes, followed by 40 cycles of 94° C for 45 seconds, annealing temperature for 45 seconds and 72° C for 45 seconds, and extension at 72° C for 10 minutes. PCR products were purified by QIAquick columns (Qiagen) and sequenced with both forward and backward primers (377 ABI Advanced Biotechnologies, Columbia, MD, USA).

Automated DNA sequencing of the *EDNRB* gene revealed a heterozygous T to C transition of codon 90 in exon 1 (Figure), which predicted a substitution of cysteine by arginine (C90R). This mutation was confirmed with backward primer for exon 1. Neither of the parents had this mutation. The mutation created a new restriction enzyme site (AciI). The mutation was absent in 100 normal unrelated Taiwanese controls by restriction fragment analysis, indicating that it was not a polymorphism. No mutation was detected in the *RET* gene of this patient.

Discussion

HSCR is a frequent neurocristopathy[10] characterized by the absence of submucosal and myenteric plexus in a variable length of the gastrointestinal tract. In the vast majority of cases (80%), the aganglionic tract involves the rectum and the sigmoid colon only (short-segment HSCR), while in 20% of cases, it extends towards the proximal end of the colon.[11] Although 80% of cases are sporadic, pedigree and segregation analyses suggested the involvement of one or several dominant genes with low penetrance in HSCR.[12] A major HSCR gene has been mapped to chromosome 10q11.2, and the disease has been ascribed to mutations in the *RET* proto-oncogene,[1,13–16] which encodes a receptor tyrosine kinase. However, the lack of genotype–phenotype correlations, the low penetrance and the sex-dependent effect of *RET* mutations supported the existence of one or more modifier gene(s) in familial HSCR.[7,17] Puffenberger et al reported evidence that HSCR type 2 (HSCR2; OMIM 600155), an apparently multigenic disorder, is due to mutations in *EDNRB*.[4]

Endothelin (EDN) is a potent vasoactive peptide, which can induce a wide range of cellular and physiologic responses.[18] In mammalian cells, there are at least two EDN receptor subtypes, EDNRA and EDNRB,[19] both of which belong to the superfamily of rhodopsin-like G-protein-coupled receptors (GPCRs)[20] that contain seven

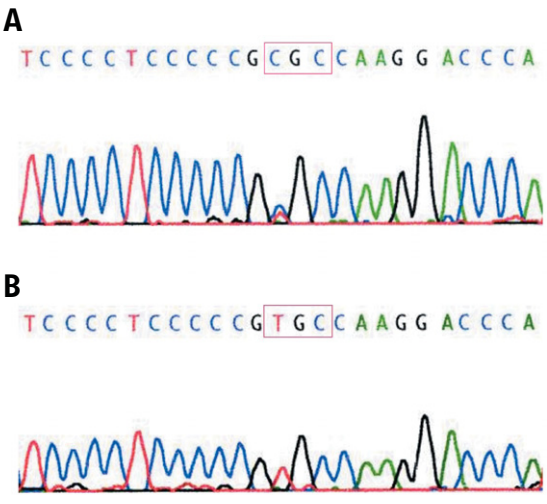

Figure. Automated DNA sequencing of the *EDNRB* gene revealed a T to C transition: (A) sequence from the proband; (B) sequence from a normal control.

transmembrane domains. The extracellular and transmembrane domains of GPCRs are involved in ligand binding, whereas the intracellular domains are involved in G protein coupling and subsequent effector regulation. To determine when EDNRB signaling is required during embryogenesis, Shin et al determined that Ednrb is required during a restricted period of neural crest development between embryonic days 10 and 12.5.[21] They concluded that EDNRB is required for the migration of both melanoblasts and enteric neuroblasts.

Arai et al demonstrated that the human genome contains a single copy of the *EDNRB* gene,[22] which spans 24 kb and comprises 7 exons and 6 introns that encode a 442 amino acid protein expressed in brain, kidney, lung, heart and endothelial cells.[23] Inagaki et al showed that this protein is also expressed in the human colon, particularly in the myenteric plexus, mucosal layer, ganglion and blood vessels of the submucosa.[24] Recently, mutations in the *EDNRB* gene have been identified in HSCR patients,[8] including deletion/insertion mutations,[25,26] non-sense mutations,[26–28] splicing mutations[29] and several missense mutations (Table 3).[4,5,9,25,30–35] The mutation was dosage sensitive in that homozygotes and heterozygotes had a 74% and a 21% risk, respectively, of developing HSCR.[4] Other analyses of patients in the extended Mennonite pedigree showed that HSCR is a multigenic disorder. For all clinical forms of HSCR, there is a greater incidence of megacolon in males than in females, and the same is true for the specific *EDNRB* mutation.[12]

The C90R mutation seems to be significant in the pathogenesis of HSCR for several reasons: (1) it was absent in 100 normal controls, making the hypothesis of a coincidental polymorphism very unlikely; (2) it led to substitution of a hydrophilic amino acid with a polar side chain (cysteine) by

Table 3. Endothelin-B receptor gene mutations in Hirschsprung disease (HSCR)/Waardenburg syndrome (WS)

Mutation		Location	Phenotype (segment length)	Genotype	Reference #
169 G>A	G57S	EC	HSCR (S)	Heterozygous	30
268 T>C	C90R	EC	HSCR	Heterozygous	Present case
325 T>C	C109R	TM I	HSCR (S)	Heterozygous	31
548 C>G	A183G	TM III	HSCR/WS	Homozygous	29
556 G>A	G186R	TM III	HSCR (L)/WS	Homozygous	33
601 C>T	R201X	IL II	ABCD syndrome	Homozygous	28
678 G>T	W226C	TM IV	HSCR (S&L)	Heterozygous	8
707 C>T	R253X	EL II	HSCR (L)/WS	Heterozygous	26
801+2 T>C	Splicing mutation	EL II	HSCR (S)	Heterozygous	28
824 G>A	W275X	TM V	HSCR (S)	Heterozygous	25
828 G>T	W276C	TM V	HSCR (L&S)	Heterozygous/homozygous	3
874 T>C	F292L	TM V	HSCR (L)/WS	Heterozygous	34
878insT	Y293L (PTC+ 6 aa)	TM V	HSCR (S)	Heterozygous	25
914 G>A	S305N	IL III	HSCR (S)	Heterozygous	24
928 G>A	A310T	IL III	HSCR (S)	Heterozygous	32
955 C>T	R319W	IL III	HSCR (S)	Heterozygous	30
1122 G>A	M374I	TM VII	HSCR	Heterozygous	4
1132delA	N378I (PTC+ 12 aa)	TM VII	HSCR (S)	Heterozygous	24
1148 C>T	P383L	TM VII	HSCR (S)	Heterozygous	30
1170 C>A	S390R	C	HSCR (L)	Heterozygous	31

ABCD = albinism, black lock, cell migration disorder of the neurocytes of the gut, and deafness; C = carboxyl-terminal region and adjacent to TM7; EC = extracellular domain; EL = extracellular loop; IL = intracellular loop; L = long-segment; PTC = premature termination of codon; S = short-segment; TM = transmembrane domain.

a basic hydrophilic amino acid (arginine); (3) this region is highly conserved between species, which probably indicates an important functional role in EDNRB signaling. The C90R mutation may change ligand binding. Functional analysis of the C90R protein will be necessary to determine the effect of the mutation on the function of the EDNRB protein.

In conclusion, the detection of a *de novo* novel mutation (C90R) of the *EDNRB* gene in our patient suggests that dysfunction of the endothelin-B receptor has a role in the etiology of some cases of HSCR, especially short-segment HSCR.

Acknowledgments

This study was supported by research grant NCKUH 93-046 from National Cheng Kung University Hospital, Taiwan.

References

1. Edery P, Lyonnet S, Mulligan LM, et al. Mutations of the *RET* proto-oncogene in Hirschsprung's disease. *Nature* 1994; 367:378–80.
2. Doray B, Salomon R, Amiel J, et al. Mutation of the RET ligand, neurturin, supports multigenic inheritance in Hirschsprung disease. *Hum Mol Genet* 1998;7:1449–52.
3. Shah KN, Dalal SJ, Desai MP, et al. White forelock, pigmentary disorder of irides, and long segment Hirschsprung disease: possible variant of Waardenburg syndrome. *J Pediatr* 1981;99:432–5.
4. Puffenberger EG, Hosoda K, Washington SS, et al. A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. *Cell* 1994;79:1257–66.
5. Hofstra RM, Osinga J, Tan-Sindhunata G, et al. A homozygous mutation in the endothelin-3 gene associated with a combined Waardenburg type 2 and Hirschsprung phenotype (Shah-Waardenburg syndrome). *Nat Genet* 1996;12: 445–7.
6. Pingault V, Bondurand N, Kuhlbrodt K, et al. SOX10 mutations in patients with Waardenburg-Hirschsprung disease. *Nat Genet* 1998;18:171–3.
7. Attie T, Pelet A, Edery P, et al. Diversity of *RET* proto-oncogene mutations in familial and sporadic Hirschsprung disease. *Hum Mol Genet* 1995;4:1381–6.
8. Chakravarti A. Endothelin receptor-mediated signaling in Hirschsprung disease. *Hum Mol Genet* 1996;5:303–7.
9. Svensson PJ, Tapper-Persson M, Anvret M, et al. Mutations in the endothelin-receptor B gene in Hirschsprung disease in Sweden. *Clin Genet* 1999;55:215–7.
10. Benish BM. The neurocristopathies: a unifying concept of disease arising in neural crest development. *Hum Pathol* 1975;6:128. [Letter]
11. Whitehouse FR, Kernohan JW. Myenteric plexuses in congenital megacolon; study of 11 cases. *Arch Intern Med* 1948;82:75–111.
12. Badner JA, Sieber WK, Garver KL, et al. A genetic study of Hirschsprung disease. *Am J Hum Genet* 1990;46:568–80.
13. Lyonnet S, Bolino A, Pelet A, et al. A gene for Hirschsprung disease maps to the proximal long arm of chromosome 10. *Nat Genet* 1993;4:346–50.
14. Angrist M, Kauffman E, Slaugenhaupt SA, et al. A gene for Hirschsprung disease (megacolon) in the pericentromeric region of human chromosome 10. *Nat Genet* 1993;4: 351–6.
15. Angrist M, Bolk S, Thiel B, et al. Mutation analysis of the RET receptor tyrosine kinase in Hirschsprung disease. *Hum Mol Genet* 1995;4:821–30.
16. Romeo G, Ronchetto P, Luo Y, et al. Point mutations affecting the tyrosine kinase domain of the *RET* proto-oncogene in Hirschsprung's disease. *Nature* 1994;367:377–8.
17. Yin L, Barone V, Seri M, et al. Heterogeneity and low detection rate of RET mutations in Hirschsprung disease. *Eur J Hum Genet* 1994;2:272–80.
18. Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev* 1994;46:325–415.
19. Vane J. Endothelins come home to roost. *Nature* 1990;348: 673.
20. Sakurai T, Yanagisawa M, Masaki T. Molecular characterization of endothelin receptors. *Trends Pharmacol Sci* 1992; 13:103–8.
21. Shin MK, Levorse JM, Ingram RS, et al. The temporal requirement for endothelin receptor-B signalling during neural crest development. *Nature* 1999;402:496–501.
22. Arai H, Nakao K, Takaya K, et al. The human endothelin-B receptor gene. Structural organization and chromosomal assignment. *J Biol Chem* 1993;268:3463–70.
23. Sakamoto A, Yanagisawa M, Sakurai T, et al. Cloning and functional expression of human cDNA for the ETB endothelin receptor. *Biochem Biophys Res Commun* 1991;178: 656–63.
24. Inagaki H, Bishop AE, Escrig C, et al. Localization of endothelin-like immunoreactivity and endothelin binding sites in human colon. *Gastroenterology* 1991;101: 47–54.
25. Auricchio A, Casari G, Staiano A, et al. Endothelin-B receptor mutations in patients with isolated Hirschsprung disease from a non-inbred population. *Hum Mol Genet* 1996;5:351–4.
26. Kusafuka T, Wang Y, Puri P. Novel mutations of the endothelin-B receptor gene in isolated patients with Hirschsprung's disease. *Hum Mol Genet* 1996;5:347–9.

27. Syrris P, Carter ND, Patton MA. Novel nonsense mutation of the endothelin-B receptor gene in a family with Waardenburg-Hirschsprung disease. *Am J Med Genet* 1999;87:69–71.
28. Verheij JB, Kunze J, Osinga J, et al. ABCD syndrome is caused by a homozygous mutation in the *EDNRB* gene. *Am J Med Genet* 2002;108:223–5.
29. Inoue M, Hosoda K, Imura K, et al. Mutational analysis of the endothelin-B receptor gene in Japanese Hirschsprung's disease. *J Pediatr Surg* 1998;33:1206–8.
30. Attie T, Till M, Pelet A, et al. Mutation of the endothelin-receptor B gene in Waardenburg-Hirschsprung disease. *Hum Mol Genet* 1995;4:2407–9.
31. Amiel J, Attie T, Jan D, et al. Heterozygous endothelin receptor B (EDNRB) mutations in isolated Hirschsprung disease. *Hum Mol Genet* 1996;5:355–7.
32. Tanaka H, Moroi K, Iwai J, et al. Novel mutations of the endothelin B receptor gene in patients with Hirschsprung's disease and their characterization. *J Biol Chem* 1998;273: 11378–83.
33. Sakai T, Nirasawa Y, Itoh Y, et al. Japanese patients with sporadic Hirschsprung: mutation analysis of the receptor tyrosine kinase proto-oncogene, endothelin-B receptor, endothelin-3, glial cell line-derived neurotrophic factor and neurturin genes: a comparison with similar studies. *Eur J Pediatr* 2000;159:160–7.
34. Boardman JP, Syrris P, Holder SE, et al. A novel mutation in the endothelin B receptor gene in a patient with Shah-Waardenburg syndrome and Down syndrome. *J Med Genet* 2001;38:646–7.
35. Pingault V, Girard M, Bondurand N, et al. *SOX10* mutations in chronic intestinal pseudo-obstruction suggest a complex physiopathological mechanism. *Hum Genet* 2002; 111:198–206.